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CAPA-gene products in the haematophagous sandfly *Phlebotomus papatasi* (Scopoli) – vector for leishmaniasis disease

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ABSTRACT

Sandflies (Phlebotominae, Nematocera, Diptera) are responsible for transmission of leishmaniasis and other protozoan-borne diseases in humans, and these insects depend on the regulation of water balance to cope with the sudden and enormous intake of blood over a very short time period. The sandfly inventory of neuropeptides, including those that regulate diuretic processes, is completely unknown. Direct MALDI-TOF/TOF mass spectrometric analysis of dissected ganglia of *Phlebotomus papatasi*, combined with a data-mining of sandfly genome 'contigs', was used to identify native CAPA-peptides, a peptide class associated with the regulation of diuresis in other hematophagous insects. The CAPA-peptides identified in this study include two CAPA-PVKs, differentially processed CAPA-PK, and an additional CAPA precursor peptide. The mass spectrometric analysis of different parts of the neuroendocrine system of the sandfly indicate that it represents the first insect which accumulates CAPA-PVKs exclusively in hormone release sites of abdominal ganglia and CAPA-PK (nearly) exclusively in the *corpora cardiaca*. Additionally, sandflies feature the smallest abdominal ganglia (~35 μm) where CAPA-peptides could be detected so far. The small size of the abdominal ganglia does not appear to affect the development of the median neurosecretory system as it obviously does in another comparably small insect species, *Nasonia vitripennis*, in which no *capa*-gene expression was found. Rather, immunocytochemical analyses confirm that the general architecture in sandflies appears identical to that of much larger mosquitoes.

1. Introduction

Water balance in insects is regulated by a number of peptide hormones, including insect kinins, corticotropin-releasing factor (CRF)-related peptides, and CAPA-peptides [2]. This regulation is of enormous importance in haematophagous insects which have to cope with the sudden and enormous intake of blood over a very short time period. The abdominal perisympathetic organs (aPSOs), the release sites of CAPA-peptides in insects, are perfectly located to allow for the regulation of the excretory and osmoregulatory Malpighian tubules. In the CAPA-precursor, different types of CAPA-peptides are encoded; namely CAPA-periviscerokinins (CAPA-PVKs) and CAPA-Pyrokinins (CAPA-PK) [17]. Both types of CAPA-peptides are known to activate different receptors in *Drosophila* [1,9,14]. Of the CAPA-peptides, only CAPA-PVKs are effective in the regulation of Malpighian tubules and it is known that CAPA-PVKs act as diuretic hormones in blood-feeding members of Diptera [2] and as anti-diuretic hormones in both blood-feeding and plant-feeding members of Heteroptera [3,4,12]. The importance of these peptide hormones for water regulation in haematophagous insects is corroborated by the unique existence of two *capa*-genes in *Rhodnius prolixus* [13], a vector for Chagas disease in humans. Both of these genes are expressed in median neurosecretory neurons of abdominal ganglia which store their secretions in the aPSOs [11].

The crucial function CAPA-peptides play in the physiology of haematophagous insects make these often species-specific peptides ideal candidates for the development of alternative strategies

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14. ABSTRACT

Sandflies (Phlebotominae, Nematocera, Diptera) are responsible for transmission of leishmaniasis and other protozoan-borne diseases in humans, and these insects depend on the regulation of water balance to cope with the sudden and enormous intake of blood over a very short time period. The sandfly inventory of neuropeptides, including those that regulate diuretic processes, is completely unknown. Direct MALDITOF/ TOF mass spectrometric analysis of dissected ganglia of Phlebotomus papatasi, combined with a data-mining of sandfly genome ?contigs?, was used to identify native CAPA-peptides, a peptide class associated with the regulation of diuresis in other hematophagous insects. The CAPA-peptides identified in this study include two CAPA-PVKs, differentially processed CAPA-PK, and an additional CAPA precursor peptide. The mass spectrometric analysis of different parts of the neuroendocrine system of the sandfly indicate that it represents the first insect which accumulates CAPA-PVKs exclusively in hormone release sites of abdominal ganglia and CAPA-PK (nearly) exclusively in the corpora cardiaca. Additionally, sandflies feature the smallest abdominal ganglia (∼35 m) where CAPA-peptides could be detected so far. The small size of the abdominal ganglia does not appear to affect the development of the median neurosecretory system as it obviously does in another comparably small insect species, Nasonia vitripennis, in which no capa-gene expression was found. Rather, immunocytochemical analyses confirm that the general architecture in sandflies appears identical to that of much larger mosquitoes.

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of insect control. The natural compounds cannot be directly used, as they are susceptible to degradation by endogenous peptidases present in the insect digestive system and circulating haemolymph [5,6]. To overcome the limitations inherent in the physical characteristics of peptides, the development of peptidomimetic analogs has become an important strategy for improving the practical potential of peptides. Peptidomimetics is a broader term used to refer to pseudopeptides and non-peptides designed to perform the functions of a peptide. Generally these peptidomimetics are derived by the structural modification of the lead peptide sequence to overcome a number of metabolic limitations, such as proteolytic degradation that restrict the use of peptides as agents capable of disrupting the life processes they regulate [23]. A prerequisite for such approaches is the knowledge of the specific sequences of mature peptides that interact with their specific receptors. From sandflies, which are responsible for transmission of leishmaniasis and other protozoan-borne diseases, no CAPA-peptides have been reported so far. Indeed, the entire neuropeptide inventory of sandflies is completely unknown. In this paper, we report the identification of the first neuropeptides of these medically important insects, focussing on *Phlebotomus papatasi*. This sandfly species is the main vector for leishmaniasis in Asia, where the majority of the world's cases of fatale visceral leishmaniasis occur. The disease is typical of a number of developing countries; Kabul (Afghanistan) has been experiencing a major epidemic, where it was estimated that 270,000 people out of a total population of 2 million in 1996 [8] have been infected. In our study, we identified, by mass spectrometry, products of the *capa*-gene of *P. papatasi* via direct analysis of the abdominal ganglia. This represented a challenge, as the tiny sand fly (1.5–3 mm) features abdominal ganglia that are extremely small (~35 μm). Subsequently, these sequences were used to identify *capa*-encoding sequences in sandfly sequence contigs. The location of *capa*-neurons in ganglia of the ventral nerve cord as well as the aPSOs was studied by means of immunocytochemistry.

2. Materials and methods

2.1. Insects

Sand flies used in this study were from a *P. papatasi* colony maintained at USDA, ARS, Knipling-Bushland U.S. Livestock Insects Research Laboratory (Kerrville, Texas). The colony was established using pupae from a long-established Israeli strain of *P. papatasi* maintained at the Division of Entomology, Walter Reed Army Institute of Research (WRAIR, Silver Spring, MA). Larvae were fed with a sand fly larval diet, a mixture of fermented rabbit feces and rabbit food [26]. Adult males were fed with 30% sucrose water and females were fed with defibrinated cattle blood using an in vitro membrane feeding system. Both larvae and adult flies were maintained at 26 ± 2 °C and a relative humidity of 85 ± 2%.

2.2. Dissection and sample preparation for mass spectrometry

Adult sandflies of both sexes were fixed with two insect pins and the wings, legs as well as the long hairs of the body wall had to be removed before the insect body was completely submerged in insect saline. This procedure prevented the development of a plastron-like air bubble around the preparation. Subsequently, the abdomen was dorsally opened with an ultrafine scissor and, after removal of the gut, the ventral nerve cord was dissected and transferred into a separate dish containing insect saline of the following composition (in mM): NaCl 126, KCl 5.4, NaH_2PO_4 0.17 and KH_2PO_4 0.22, pH 7.4. The aPSOs were so small that they could not be visualized with high resolution stereomicroscopes (SteREO Discovery.V8 and Lumar.V12; Carl Zeiss, Göttingen, Germany) which we used for sandfly preparations. Therefore, we directly profiled the abdominal ganglia (~35 μm) for CAPA-peptides. Ganglia were cut into a dorsal and a ventral piece, respectively, and transferred with a glass capillary in a drop of purified water on the sample plate for MALDI-TOF mass spectrometry. The water was removed using the transfer glass capillary and the tissues were air-dried. For peptide analysis, a limited amount of matrix solution (α-cyano-4-hydroxycinnamic acid dissolved in methanol/water 1:1 v/v) was pumped on the dried samples. Each preparation was allowed to dry again and then covered with pure water for a few seconds, which was finally removed by cellulose paper.

2.3. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry

Mass spectrometric analysis was performed either on an ABI 4800 proteomics analyzer (Applied Biosystems, Framingham, MA) or an UltrafleXtreme mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany). All acquisitions were taken in manual mode. Initially the instruments were operated in reflectron mode, in order to determine the parent masses. For the tandem MS experiments, we used PSD as well as CID mode. The fragmentation data obtained in these experiments were analyzed with the respective software packages (Data Explorer 4.3 or flexAnalysis 3.3) and used to determine the sequences of the sandfly neuropeptides. A detailed description of sample preparation and mass spectrometry analysis of nervous tissue from small insect species is given in [25].

2.4. Homology based search of sandfly databases

The genomic sequence of *P. papatasi* was screened with the amino acid sequence RSGNMGLFPFPRVGR using TBLASTN. The genomic data were produced by The Genome Institute at Washington University School of Medicine in St. Louis and can be obtained from ftp://genome.wustl.edu/pub/organism/Invertebrates/Phlebotomus_papatasi/assembly/Phlebotomus_pap. The hit on contig21111 was further analyzed using CLC Main Workbench 6.2 (http://www.clcbio.com) and gene prediction programs (http://linux1.softberry.com/berry.phtml). Peptide processing was predicted using the ProP server (http://www.cbs.dtu.dk/services/ProP/).

2.5. Immunocytochemistry

Ventral nerve cords were fixed for 3 h at 4 °C with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2. Subsequently, samples were washed 3 times at 15 min in PBS at room temperature. The preparations were then incubated for 2 days at room temperature in anti-CAPA-PVK serum (1:4000) with an antiserum, which was raised against *Periplaneta americana* CAPA-PVK-2 (GSSSGLISMPRV-NH_2) [15], diluted with PBS containing 0.25% bovine serum albumin and 10% normal goat serum. Following washing in PBS for 24 h, the preparations were incubated in the secondary antibody conjugated to Cy3 (1:3000; Amersham, Braunschweig, Germany) in PBS containing bovine serum albumin (2.5 mg/ml) for 2 days at room temperature. Finally, the preparations were washed again for 24 h in PBS and mounted in Mowiol (Calbiochem, Darmstadt, Germany).

2.6. Documentation

Immunostainings were examined with a confocal laser scanning microscope (ZEISS LSM 510 Meta system, Jena, Germany), equipped with a Plan-Neofluar 40×/1.3 oil objective and a HeliumNeon1 laser (wavelength 543 nm). Serial optical sections were assembled into

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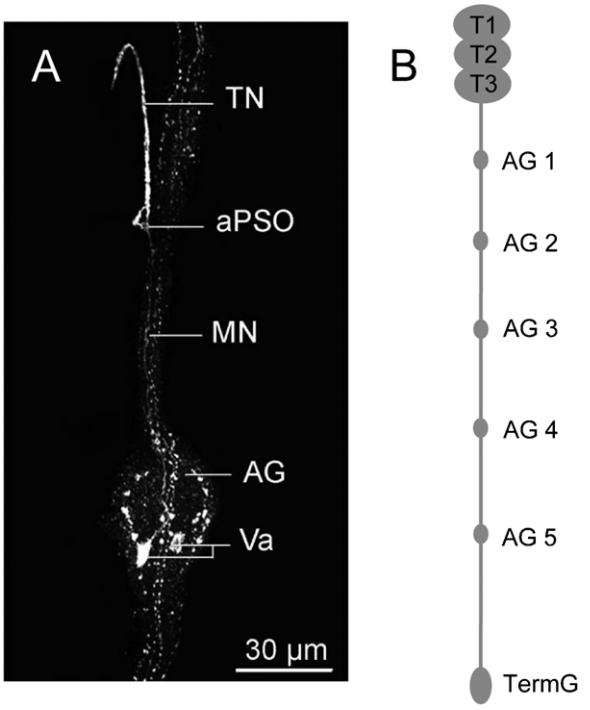

Fig. 1. (A) Anti-Pea-PVK-2 immunofluorescence staining in a whole-mount preparation of an abdominal ganglion of the sandfly, *P. papatasi*. Two Va-neurons have projections into the median nerve; the aPSO is strongly stained. Additional immunoreactive fibres in the connectives and lateral in the ganglion originate from cells in the subesophageal ganglion that express pyrokinins. (B) Schematic overview of the sandfly ventral nerve cord. AG, abdominal ganglion; aPSO, abdominal perisympathetic organ; MN, median nerve; TN, transverse nerve; Va, ventral abdominal *capa*-neurons.

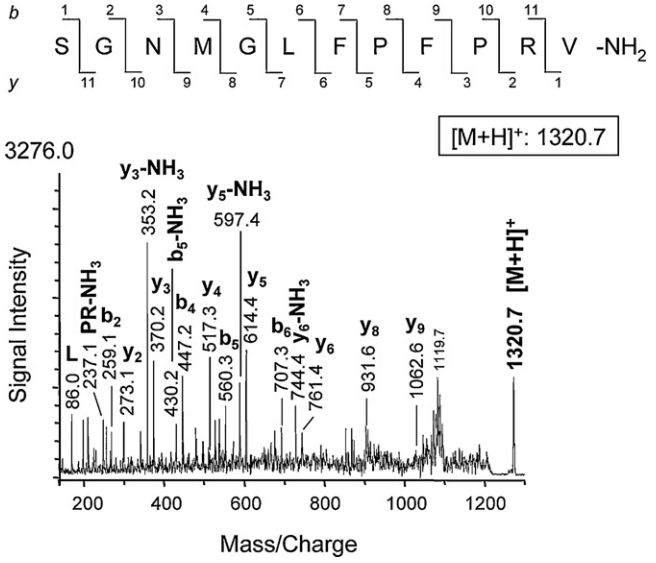

Fig. 2. MALDI-TOF/TOF tandem mass spectrum of the peptide at *m/z* 1320.7 under conditions of high collision energy. Prominent y- and b-type fragments are labeled. Fragments were analyzed manually with the aid of sequence information from related dipteran species and the resulting sequence shows a CAPA-PVK. For assignment of internal Leu see Fig. 3.

combined images. Images were exported and processed with Adobe Photoshop 7.0 software.

3. Results

3.1. Location of capa-neurons in the ventral nerve cord

The ventral nerve cord of *P. papatasi* consists of three fused but distinct thoracic ganglia, five unfused abdominal ganglia, and the terminal ganglion. Since the first abdominal neuromere is fused with the metathoracic ganglion, the terminal ganglion is the fusion product of the abdominal neuromeres 7–11. Immunostaining with the anti-CAPA-PVK serum revealed two immunoreactive ventral median cell bodies posterior in each of the unfused abdominal ganglia (Fig. 1). Axons of these neurons run anterior and develop a loop before leaving the ganglion via the median nerve. PVK-immunoreactive fibres exit the median nerve at the origin of the transverse nerves; in insects this junction is usually referred to as aPSO. The first neuromere of the terminal ganglion also develops *capa*-neurons with axons into aPSO-6.

3.2. Identification of capa-gene products by MALDI-TOF mass spectrometry

Since the putative release sites of CAPA-peptides (aPSOs; see Fig. 1) are diminutive, we used randomly dissected pieces of the ventral part of abdominal ganglia for direct mass spectrometric peptide profiling. Prominent ion signals in the *m/z* range 900–2500 were fragmented from these samples and the resulting fragment spectra were screened for ions typical of the C-terminus of CAPA-PVKs (e.g. at *m/z* = 370/353 representing the y_3/y_{3-17} fragment pair of PRV-NH_2). A distinct hit was found in a peptide with *m/z* 1008.6 ([M+H]$^+$). Subsequently, all spectra containing this peptide were analyzed and ions at *m/z* 1025.6, 1320.7, 1724.9, and 2013.1 ([M+H]$^+$) were found accompanying this putative CAPA-PVK in all spectra. Fragment ions obtained from these peptides suggested additional CAPA-PVKs (1025.6, 1320.7). In a next step, we focused on these peptides and obtained a number of spectra where CAPA-PVKs generated the most abundant ion signals. A considerable number of fragmentations from these selected samples were necessary to obtain the complete sequences of two CAPA-PVKs of *P. papatasi* (1008.6, 1320.7; see Fig. 2). As it turned out, the substance at *m/z* 1025.6 is *Phlebotomus*-CAPA-PVK-2 with C-terminal Gln instead of pGlu.

3.3. BLAST-search

The obtained sequence information was used to perform TBLASTN searches in genomic *P. papatasi* databases. This resulted in the identification of a partial *capa*-gene on contig21111 containing two exons (Fig. 3); encoding the prohormone containing CAPA-PVK-1, CAPA-PVK-2, a precursor peptide located between CAPA-PVK-2 and CAPA-PK (CAPA precursor peptide B; CPPB; see [24]), and CAPA-PK. The N-terminal sequence of the preprohormone including the signal peptide could not be found on this contig.

3.4. Re-evaluation of MS-spectra using the sequence information obtained from the CAPA-precursor

The (partial) CAPA-precursor sequence contains four peptides flanked by mono- or dibasic cleavage sites; the two already sequenced CAPA-PVKs, a CAPA-PK, and CPPB. Identical mass ions with CPPB (1724.9) and occasionally CAPA-PK (1557.8) in mass spectra of aPSOs indicate the expression of all predicted peptides from the CAPA-precursor, but the signal intensity of CAPA-PK was extremely low (Fig. 4). We found, however, that the prominent ion signal at *m/z* 2013.1 is mass-identical with a C-terminally extended CAPA-PK which is not cleaved at the expected monobasic cleavage site but at the following dibasic cleavage site (see Fig. 3). Ion intensity of this larger peptide was not sufficient to reveal an unambiguous fragment series. Interestingly, the CAPA-PK, which did not

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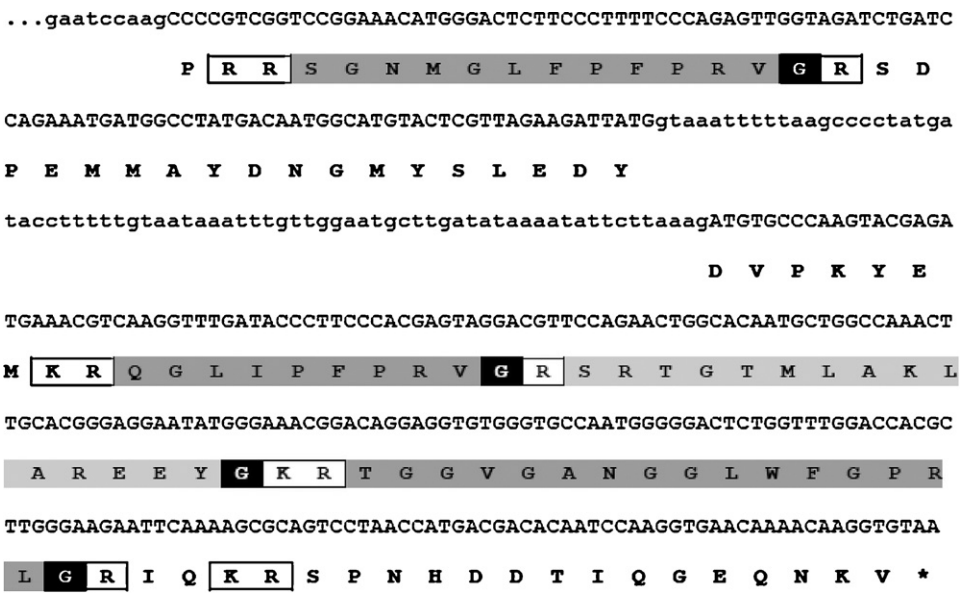

Fig. 3. Partial sequence of the translated *Phlebotomus papatasi capa*-precursor found on contig21111 (pos. 955-1373). Introns are given in lowercase, exons in uppercase, the translated protein sequence in bold. The CAPA-PVK-1, CAPA-PVK-2 and predicted CAPA-PK sequences are highlighted in dark grey, the CPPB sequence in light grey. Mono- and dibasic prohormone convertase cleavage sites are boxes, glycines used for amidation are highlighted in white on black ground. The stop codon is shown by a star.

Table 1
List of conserved CAPA-peptides (receptors known in *D. melanogaster*) that were identified from *P. papatasi* (this study), other dipteran species, and a cockroach (*Periplaneta americana*). *D., Drosophila*; *S., Sarcophaga* (=*Neobellieria*); *M., Musca; A. aegypti, Aedes aegypti; A. gambiae; Anopheles gambiae.*

Species	CAPA-PVK-1	CAPA-PVK-2	CAPA-PVK-3	CAPA-PK
Diptera				
Brachycera				
D. melanogaster	GANMGLYAFPRVa	ASGLVAFPRVa	–	TGPSASSGLWFGPRLa
D. virilis	GANMGLYTFPRVa	ASLVPFPRVa	–	TGPSASSGMWFGPRLa
S. bullata	NGGTSGLFAFPRVa[a]	AGLLVYPRLa	–	AGPSATTGVWFGPRLa
M. domestica	AGGTSGLYAFPRVa	ASLFNAPRVa	–	AGPSATTGVWFGPRLa
Delia radicum	GGGG**T**SGLFAFPRVa	**A**G**L**F**AQ**PRLa		**A**GPSATTGVWFGPRLa
Nematocera				
A. aegypti	GPTVGLFAFPRVa	pQGLVPFPRVa	–	AGNSGANSGMWFGPRLa
A. gambiae	GPTVGLFAFPRVa	pQGLVPFPRVa	–	AGGTGANSAMWFGPRLa
P. papatasi	SGNMGLFPFPRVa	pQGLIPFPRVa	–	TGGVGANGGLWFGPRLa
Blattodea				
P. americana	GASGLIPVMRNa	GSSGLISMPRVa	GSSSGLISMPRVa	GGGGSGETSGMWFGPRLa

[a] Recent analyses of CAPA-peptides from related *D. radicum* [27] and *Lucilia cuprina* [21] suggest that the N-terminal Asn has to be replaced by Gly-Gly.

occur together with CAPA-PVKs but CPPB, was detected in preparations of the posterior *corpora cardiaca*/anterior part of the aorta (Fig. 5), providing support for the expression of this peptide as well. A summary of the CAPA-peptides of *P. papatasi* as well as a comparison of these peptides with homologous peptides from other Diptera is given in Tables 1 and 2.

4. Discussion

The sequences of CAPA-peptides from sandflies, which were identified in this study, represent the first information on neuropeptides of these tiny, medically important insects. As in many other insects [17], the *capa*-gene of *P. papatasi* encodes for two PVKs and a single PK. The sequences are not strikingly different from the CAPA-peptides of other Diptera species. In contrast to *Drosophila*-CAPA-PVKs and all other known CAPA-PVKs from brachyceran flies, the CAPA-PVK-2 of *P. papatasi* and other nematoceran flies have the N-terminus of CAPA-PVK-2 blocked by pyroglutamate (see Table 1). Pyroglutamate may prevent rapid degradation of this peptide hormone in the haemolymph. The ratio of blocked/non-blocked CAPA-PVK-2 ranged between 70/30 and 40/60; likely the result of spontaneous cyclization of Gln. Other peptide hormones of the sandfly with N-terminal Gln (e.g. corazonin, see Fig. 5) show complete cyclization.

Table 2
Complete list of CAPA-peptides processed from the *capa*-precursor of the sandfly *P. papatasi* and confirmed by MALDI-TOF mass spectrometry.

Peptide name	Peptide sequence	$[M+H]^+$	MS	MSMS
CAPA-PVK-1	SGNMGLFPFPRVa	1320.6881	+	+
CAPA-PVK-2 (pQ)	pQGLIPFPRVa	1008.6255	+	+
CAPA-PVK-2 (Q)	QGLIPFPRVa	1025.6255	+	+
CPPB	SRTGTMLAKLAREEYa	1724.9112	+	+
CAPA-PK	TGGVGANGGLWFGPRLa	1557.8285	+	–
ext. CAPA-PK	TGGVGANGGLWFGPRLGRIQ-OH	2013.0777	+	–

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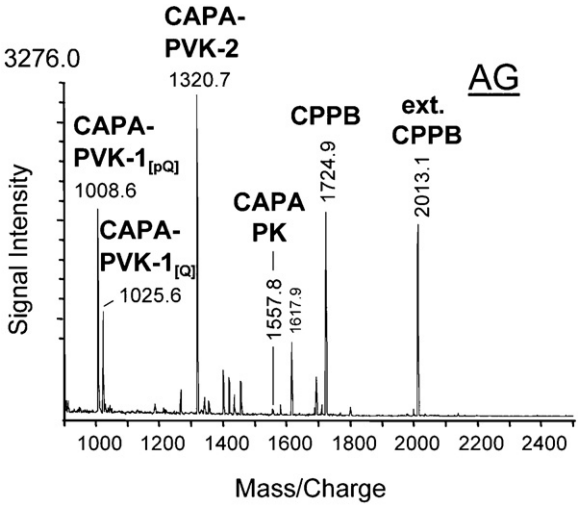

Fig. 4. MALDI-TOF mass spectrum (*m/z*: 900–2500) of an abdominal ganglion preparation of *P. papatasi*. Ions with high signal intensity represent CAPA-peptides. Note, that only traces of the predicted CAPA-PK are detectable. On the other hand, the prominent ion signal at *m/z* 2013.1 is mass-identical with the C-terminally extended form of CAPA-PK, which suggests that the predicted C-terminal monobasic cleavage site is not used in neurosecretory cells of abdominal ganglia.

The location of the *capa*-expressing neurons in abdominal ganglia of the sandfly closely resembles the distribution of homologous neurons in *Aedes aegypti* [20]. A pair of ventral abdominal neurons (Va-neurons) of the five abdominal ganglia and the first neuromere of the terminal ganglion show projections in aPSOs. In *Drosophila*, only three neuromeres develop homologous *capa*-neurons [22]. Mass spectra verified that the *capa*-neurons in abdominal ganglia process CAPA-PVKs and CPPB but little or no CAPA-PK. Vice versa, mass spectra obtained from the part of the retrocerebral complex where nerves from the *corpora cardiaca* invade the anterior part of the aorta, indicate the presence of CAPA-PK and CPPB but no traces of CAPA-PVKs or extended CAPA-PK were detected in this neurohemal area. Thus, only CPPB is abundant in both tissues and thereby confirms that the CAPA-PK signal in the retrocerebral complex is not a simple mass-match only.

Given the fact that CAPA-PK and CAPA-PVKs activate different receptors, this differential expression/processing is likely an advanced stage of functional differentiation. In insects, all intermediate stages of this differential expression/processing have been found [10,17]. First, in basal pterygota such as cockroaches and locusts, CAPA-peptides (PVKs and PK) are exclusively stored in aPSOs but not in the *corpora cardiaca*; obviously no differential expression and/or processing occur [see 18]. In these insects, another gene (*pyrokinin/fxprl/pban*-gene) encodes peptides very similar with CAPA-PKs and these peptides are synthesized in the subesophageal ganglion (SEG) and stored in the *corpora cardiaca*. Second, in *Manduca sexta* (Lepidoptera) all predictable neuropeptides of the *capa*-gene were found in aPSOs as well as in *corpora cardiaca* [10]. Hence, no differential expression/processing of CAPA-peptides were found but additional expressing and release sites of all CAPA-peptides exist. Notably, the additional expression of CAPA-peptides in the SEG of *M. sexta* occurs in neurons of the labial neuromere that co-express PKs from the *pyrokinin/fxprl/pban*-gene which are sequence-related with CAPA-PK (see above for locusts/cockroaches). Third, species such as *D. melanogaster* [16], *A. aegypti* [20], and *R. prolixus* [11] synthesize all CAPA-peptides in neurosecretory cells of abdominal ganglia but in cells of the SEG only the CAPA-PK is detectable. In these species, the *pyrokinin/fxprl/pban*-gene does not contain a CAPA-PK-related peptide anymore. With the sandfly *P. papatasi* we found for the first time an insect which processes CAPA-PVKs exclusively in abdominal ganglia and a CAPA-PK (nearly) exclusively in neurons of the subesophageal ganglion that provide the *corpora cardiaca* with neurosecretions. The absence/very low abundance of CAPA-PK in abdominal ganglia obviously results from a poor recognition of the monobasic cleavage site (Arg) at the N-terminus of the CAPA-PK sequence in the precursor. Instead, the following dibasic cleavage site (KR) is used and this cleavage ends in an extended CAPA-PK which is likely biologically inactive.

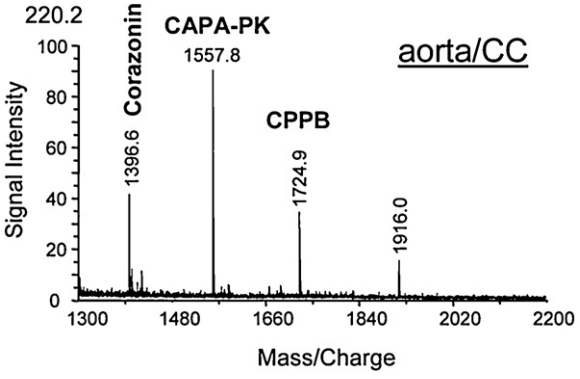

Fig. 5. MALDI-TOF mass spectrum (*m/z*: 900–2500) of a preparation of the posterior *corpora cardiaca*/anterior part of the aorta. Prominent ion signals mass-identical with CAPA-PK and CPPB are detectable but no trace of CAPA-PVKs. The signal at *m/z* 1396.6 was fragmented and represents a rare corazonin form only known from a single crane fly (Tipulidae) species (pQTFQYSRGWQN-NH_2) [19]; the remaining signals could not be assigned.

Sandflies feature the smallest abdominal ganglia where insect CAPA-peptides could be detected so far. In other comparably small species such as *Nasonia vitripennis*, the *capa*-neurons in abdominal ganglia are absent [7], although closely related but larger parasitic wasps express CAPA-peptides (Rodewald, Predel; unpublished). Thus, miniaturization does not necessarily affect the development of the median neurosecretory system in insects; in sandflies its general architecture is identical with that of the much larger mosquitoes.

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